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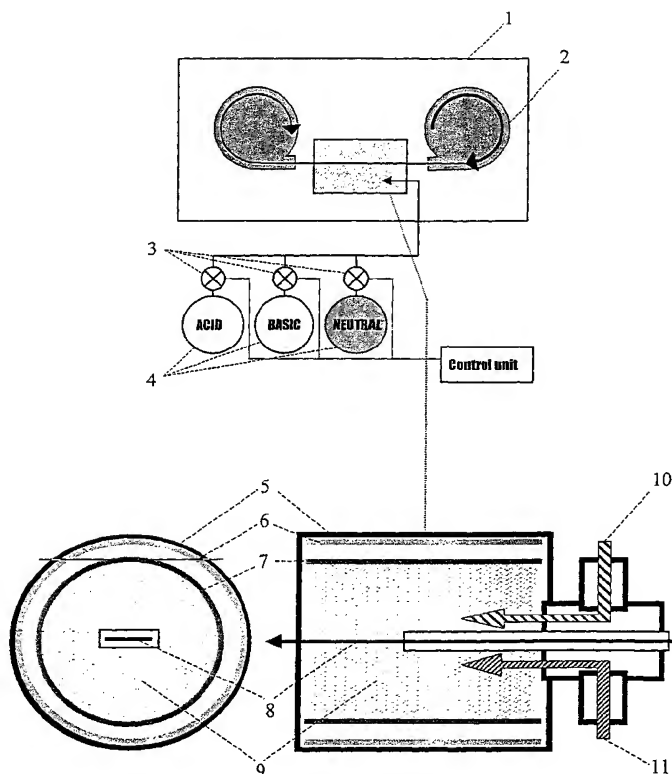
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(54) Title: A PROCESS FOR THE SEPARATION OF BIOCOMPONENTS ON A SUBSTRATE SURFACE



(57) Abstract: The invention relates to a process for the separation of biocomponents, such as tissue, cells, body fluids, proteins, nucleic acids etc. The process comprises the steps of: i. providing a material comprising a substrate that has a surface separation layer on at least a part of its surface; ii. providing a liquid comprising the biocomponents; and iii. contacting the liquid comprising the biocomponents with the surface separation layer. The surface separation layer may preferably comprise a stepwise or continuous gradient in at least one direction along the surface of the substrate. The gradient may include a change in e.g. the thickness, the cross-linking degree, the density or the molecular weight. The gradient may alternatively include a change in the concentration of one or more components or in the type of components, so that at least one property, such as hydrophilicity or pH value, is changing continuously or stepwise. An electrophoresis technique may be applied to the method.

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A PROCESS FOR THE SEPARATION OF BIOCOMPONENTS ON A SUBSTRATE SURFACE

FIELD OF THE INVENTION

The present invention relates to a novel process for the separation or isolation of
5 biocomponentes, in particular biomolecules, such as proteins. The process makes use of
a material comprising a sheet-like substrate with a coating which may represent a
gradient e.g. a composition gradient.

BACKGROUND OF THE INVENTION

Separation of proteins from a complex mixture has traditionally been performed by
10 utilising chromatographic techniques or gel electrophoresis techniques. Traditional gel
electrophoresis techniques are however time and labour consuming and may involve
limitations with respect to resolution.

pH gradients in gels have e.g. been provided for polyacrylamide matrices as described in
WO 93/11174 and WO 97/16462.

15 Since 1975, complex mixtures of proteins have generally been separated by means of two
dimensional gel electrophoresis in which the physical separation of the proteins in the first
dimension gel is based upon a separation according to the isoelectric point of each of the
proteins to be analysed. This is referred to as isoelectric focussing (IEF) of the proteins.
(see e.g. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol
20 Chem. 1975 May 25;250(10):4007-21).

However, a single IEF gel cannot resolve all of the proteins present in a single cell type
since there are in excess of typically 20,000 different proteins in a cell. Therefore many
investigators who want to study and identify some or all of the proteins expressed in a cell
(proteomics) have used a second 'dimension' – a second gel wherein the proteins are
25 separated at right angles to the first IEF gel, where the proteins are separated based on
differences of their respective molecular weight. This is called two-dimensional gel
electrophoresis (2DGE).

The objective of the invention is to provide an alternative process of separating
biocomponents such as biomolecules, by use of which a high resolution can be obtained.

Another objective is to provide a process of separating biocomponents such as biomolecules which can be used for separating biocomponents e.g. proteins from compositions comprising a large amount of different biocomponents e.g. above 5,000, or above 10,000 or even above 15,000 different biocomponents.

- 5 Yet another objective is to provide a process of separating and optionally identifying biocomponents which is relatively simple and easy to carry out, and which is preferably highly reproducible.

A further objective of the invention is to provide a process of separating biocomponents by use of which a high resolution can be obtained, and which process is labour-saving

- 10 compared to known processes.

These and other objectives have been achieved by the invention as defined in the claims.

- The invention relates to a process for the separation of biocomponents, wherein the biocomponents, or at least some of the biocomponents in a sample are separated and optionally isolated using as sheet-like substrate which may e.g. have a gradient, such as a
15 composition or a structure gradient on at least a part of the surface thereof. The gradient may be graduating continuously or stepwise in at least one direction along the surface of the substrate.

DESCRIPTION OF THE FIGURES

- Figure 1 illustrates a plasma reaction chamber which is useful for the preparation of one
20 type of sheet-like material to be used in the process according to the invention.

Figure 2. illustrates an example of a voltage and current profile of the embodiment exemplified in example 4.

Figure 3. illustrates an example of a voltage and current profile of the embodiment exemplified in example 5.

25 DETAILED DESCRIPTION OF THE INVENTION

The present invention is intended for the separation of biocomponents. When used herein, the term "biocomponents" is intended to include components of biological origin, such as human origin or synthetic components resembling these. The biocomponent may

e.g. include biomolecules, tissues, cells, body fluids, blood components, microorganism, and derivatives thereof, or parts thereof as well as any other biocomponent.

The biocomponent may include one or more biomolecules of microbial, plant, animal or human origin or synthetic molecules resembling them. The biocomponent or components
5 may preferably be of human origin or synthetic molecules resembling them

Basically the process is particularly useful for the separation of biomolecules such as proteins, nucleic acids, such as RNA, DNA, PNA, oligonucleotides peptides, hormones, antigen, antibodies, and complexes including one or more of these molecules, said biomolecule preferably being selected from the group consisting of proteins and protein
10 complexes.

Particularly relevant examples of biomolecules are proteins, peptides and protein complexes. Protein complexes include any chemical substances wherein at least one protein is linked, e.g. linked by ionic links or Van der Waals forces. The protein complexes may e.g. include at least 10 % by weight of the protein.

15 The proteins include denatured, partly denatured and non-denatured proteins. The denaturation degree depends largely on the substrate, the composition or composition gradient on the substrate, as well as the liquid comprising the proteins. Thus in some of the embodiments, non-denatured proteins can be separated, because the biomolecules are adsorbed to (and are mobile on) the surface of the substrate. This provides the further
20 advantage that separated proteins or other biocomponents can be tested directly for biological activity without the need for an isolation and optional re-folding step.

The invention is based on the idea of separating organic compounds, namely biocomponents on a substrate comprising a surface separating layer, wherein this surface separating layer is relatively thin, i.e. the surface separating layer may e.g. have a
25 thickness of 1, 2, 5, 10 or 50 or even up to about 10,000 molecular layers of the molecules constituting the surface layer. the number of molecule layers may be detected using common methods of measuring thickness and or mass of the separating layer and calculating the average number of molecule layers. In order to have an optimal resolution, the number of functional groups should preferably be sufficiently large, and thus the
30 surface separating layer may e.g. have a thickness of up to about 20 μm . However, it should be observed that a too thick layer may give prolonged separation time, and further the amount of the sample should be increased as well or the resolution may be poor. This

feature will be described further later on. Preferably the thickness of the separating layer is between 0.01 and 15 μm , such as between 0.5 and 10 μm . The larger the surface area coated with the separating layer, the thinner the separating layer may be.

In the first characterising step of the process of the invention, a material having a sheet-
5 like substrate is provided.

The sheet-like substrate may in principle have any shape e.g. the shapes described in PCT/DK01/00689, which is hereby incorporated by reference.

The term sheet-like substrate includes any substrate having a 3-dimensional shape, length, thickness and width, wherein the substrate in at least one of its dimensions,
10 designated the length and measured at its longest point, is more than, preferably more than 10 times, more preferably more than 100 times its shortest dimension, designated its thickness and measured in its shortest point. Preferably the substrate in its dimension designated its thickness and measured at its shortest point, is less than 0.5 times its other 2 dimensions measured at their longest points, preferably less than 0.1 times its other 2
15 dimensions. The sheet-like substrate preferably includes tapes, bands, strips, felts, sheets, non-woven structures, woven structures, membranes, films, plates, etc. having regular or irregular dimensions. In one particularly interesting embodiment, the sheet-like substrate is a tape roll, which can have a length of up to several meters. The sheet-like substrate may also include a hollow pipe with an inner surface and an outer surface or be
20 in the form of a cord or a bundle of cords.

The width and the thickness are normally of about the same order of magnitude. As an example, the thickness may be in the range of 50-500 μm whereas the width may be in the range of 3-300 mm.

In one specific embodiment, the sheet-like substrate is in the form of a three dimensional
25 unit, wherein one dimension designated the length is more than 2 times, preferably more than 5 times and even more preferably more than 10 times longer than the longest of the other two dimension, preferably the length is at least 10 cm such as 25 or 50 cm, more preferably at least 100 cm and even more preferably at least 200 cm.

Generally it is preferred that the shortest dimension designating the thickness is between
30 1 μm and 10 mm, more preferably between 50 and 500 μm . The dimension designating

the width may preferably be between 1 μm and 1000 mm, more preferably between 3 and 300 mm.

In one embodiment, the sheet-like substrate is in the form of a cord, said cord preferably having a round or angular cross-section, such as triangular or rectangular, the cord
5 comprising a coating i.e. a separation layer on its surface extending along the whole or part of the length of the cord and the coating preferably constituting a surface composition gradient or a structure gradient in one direction essentially following the length of the cord. Preferably the cord has a substantially circular cross-section with a diameter of 0,1-10 mm, preferably between 1 and 4 mm.

- 10 In the embodiment of the first group of preferred embodiments, where the sheet-like substrate is in the form of a hollow pipe, it is preferred that the inner surface of the hollow pipe is coated with a gradient coating such as a polymer gradient coating.

The substrate may be of any material e.g. it may be of materials capable of absorbing liquid or it may be non-adsorbing e.g. in the form of non-porous glass. Absorbing
15 substrates may be further divided into substrates wherein the liquid is migrated into and optionally chemically bound in the material and porous substrates such as non-woven felts where the water is absorbed into the capillaries of the materials. In both situations it may be desired to wet the substrate prior to the application of the liquid containing the biocomponents to be separated in order to reduce the amount of the liquid with
20 biocomponents necessary. Thereby non-specific bonding to molecules or components may also be reduced. Generally substrates that absorb large amounts of liquid i.e. such as 100 % of the weight of the substrate or more due to migration into and optionally chemically binding in the material should be avoided.

The substrate may be a layered material comprising layers of one or more materials, such
25 as layered materials. Useful materials include glass, glass-fiber based materials, metals, solid or foamed polymers, non-woven or woven polymers, paper, fibres, such as carbon fibres; aramide fibres; fiberreinforced materials; ceramics; or mixtures or combinations thereof.

The polymer materials may include one or more polymers selected from the group
30 consisting of polyolefins including polyethylene (PE) and polypropylene (PP); polyesters; polytetrafluoroethylene (PTFE); tetra-fluoroethylene-hexafluoropropylene-copolymers (FEP); polyvinyl-difluoride (PVDF); polyamides; polyvinylchloride (PVC), rubbers such as

silicon rubbers and mixtures thereof. Generally it is preferred to use non-woven felt made from polymer fibres. This is in the following referred to as felt.

The material should comprise a sheet-like substrate which has a surface separating layer on at least a part of the surface thereof. The surface separating layer may or may not
5 comprise or e.g. be constituted by a gradient. Basically it is preferred that the surface layer comprise a gradient, as a gradient may provide an improved resolution and further may give rise to improved isolation of selected biocomponents.

A gradient is generally defined as a stepwise or continuous, graduating change of the layer composition or structure in at least one direction along the surface of the substrate.
10 The substrate may comprise two or more gradients e.g. as described in PCT/DK01/00698.

The gradient may be a structure gradient in the form of a stepwise or continuously graduating structure change selected from the group consisting of change in thickness, change in cross-linking degree, change in density and change in molecular weight.

15 In its simplest aspect the gradient is a composition gradient. The term composition gradient thus means that the surface has a variation in chemical composition varying along the surface in a graduating pattern, preferably so that the composition in the form of a mixture of two or more compounds is varied with respect to the amount of the respective compounds stepwise or continuously along the surface of the substrate, preferably in one
20 or more defined directions. The surface preferably has an attractive force variation along the surface in a stepwise or continuously graduating pattern, where an attractive force variation means a variation in the surface attractive force property relative to organic compounds, such as a variation in pH value, affinity for particular epitopes, binding partners or ligands for the biocomponent or a variation in hydrophobicity/hydrophilicity,
25 preferably the gradient is a pH gradient or a hydrophobicity gradient.

Thus, generally the composition gradient may include a stepwise or continuously graduating composition change selected from the group consisting of change of the concentration of one, two or more components, change of type of components so that at least one property, such as hydrophilicity or pH value is changing continuously or
30 stepwise.

The term "surface composition gradient" preferably includes the surface separation layer on the substrate into which the biocomponents are able to penetrate under influence of the composition gradient during the step of separation. The affinity of the surface layer may be modulated by varying the amount of liquid applied to the surface, until the surface
5 layer is completely moisturised.

The surface composition gradient thus means that the surface has a variation in chemical composition varying along the surface including the surface layer capable of adsorbing the liquid in a graduating pattern, preferably so that the composition is varied with respect to the concentration of one or more components and/or in the form of a mixture of two or
10 more compounds which is varied with respect to the amount of the respective compounds stepwise or continuously along the surface of the substrate.

The composition gradient is preferably in the form of a polymeric coating, wherein the term "polymeric" means that the coating or separating layer comprises at least 10 %, preferably at least 50 % by weight of a polymer in dry state. The polymeric coating may
15 preferably be crosslinked e.g. in a graduating manner so that the number of crosslinks varies to provide a structure gradient; or in a non-graduating manner. The composition variation may be a variation of the polymeric units or e.g. a variation of other components in the polymeric compositions, such as functional groups linked to the polymer or component comprising functional groups linked, adhered to the polymer or entrapped in
20 the polymeric matrix.

It should be noted that the term "functional groups" includes charged groups, groups which modify the surface with respect to hydrophility/hydrophobicity, and groups that are reactive towards or attract the biocomponents to be separated.

In one embodiment, the gradient may be a composition gradient in the form of a
25 separation layer comprising a ligand with a functional group, which functional group may be as defined above. The gradient is constituted by a change of the number or type of ligands. The ligands may be any type of ligand, and it may be linked to the surface or to a primer layer on the surface by use of any means, such as covalently linking by use of a thermoreactive or photoreactive linking. The linking may e.g. be performed via a spacer.
30 Further information about how such a linking may be performed can be found in WO 9631557 and WO 0104129, which are hereby incorporated by reference. A preferred method of linking the ligand to the substrate e.g. via a spacer includes quinones e.g. anthraquinones.

Generally the surface separation layer, e.g. in the form of or comprising a gradient, may include one or more of the components acids, such as organic acids, amino acids, lipid acids and poly acids thereof; bases such as organic bases, amino acids and poly bases thereof; aromates, metal components, such a organometals, halogens, zwitter ions, 5 ampholines, antigens and antibodies. These components may e.g. be linked or adhered to the substrate, linked or adhered to a polymeric layer on the substrate, or embedded in a polymeric matrix.

It is preferred that the gradient coating constitutes a surface composition and/or structure gradient in one single direction along the surface of the substrate. This surface gradient 10 may be in one direction which preferably is a direction following the length direction of the sheet-like substrate, such as a direction substantially parallel to the longitudinal axis of the sheet-like substrate. Alternatively the substrate may comprise a gradient in two or more directions essentially parallel to the plane of the surface of the substrate, e.g. the surface separation layer may comprise or constitute gradients extending radially from a central 15 area or a gradient extending in two opposite directions.

In one embodiment, the substrate comprises a composition and/or structure gradient, which constitutes two or more surface gradients, such as gradients following each other along the same single direction or different gradients in different directions. The two or more surface gradients may e.g. be pH gradients with different pH separation values.

- 20 The variation in composition may for example represent a pH gradient, a hydrophobicity gradient, a polarity gradient, a surface tension gradient, a biocompatibility gradient, a binding affinity gradient, etc. A gradient of metal concentration e.g. in the form of organo metallic components may also be accomplished. A pH gradient is particularly relevant where the material is utilised in a process for the separation of proteins.
- 25 The gradient is generally in a direction substantially parallel to the plane of the material or sheet-like substrate. The "plane of the material or sheet-like substrate" refers to the macroscopic plane of the sheet, film, tape, etc. Thus, normally (in particular for films, strips and tapes) the gradient may as mentioned preferably be in a direction substantially parallel to the longitudinal axis of the material.
- 30 The material according to the invention may be constituted of the sheet-like substrate inclusive the polymer gradient coating and any pre- or top-coating.

Alternatively the material according to the invention may, besides the sheet-like substrate inclusive the polymer gradient coating and any pre- or top-coating, comprise a supporting element for the substrate. The supporting element may in principle be any type of supporting element which does not destroy or totally mask the polymer gradient coating. It is preferred that the supporting element is shaped so that the total material preferably is essentially sheet-like, wherein the term sheet-like material means a material having a shape as a sheet-like substrate as defined above.

Generally it is preferred that the supporting element is in the form of a supporting sheet selected from the group consisting of polymers, such as polyolefins including polyethylene (PE), and polypropylene (PP); polyester; polytetrafluoroethylene (PTFE); tetrafluoroethylene-hexafluoropropylene-copolymers (FEP); polyvinylidene fluoride (PVDF); polyamides; polyvinylchloride (PVC); rubbers such as silicon rubbers; glass; paper; carbon fibres; aramide fibres; ceramics; metals or mixtures or combinations thereof.

Preferred material for the process

According to the invention it is generally preferred to apply a material comprising a composition gradient as disclosed above and as further described in the following. The material may be as disclosed in PCT/DK01/00689. The plasma method used and the component applied may be modified e.g. by using plasma methods disclosed in PCT/DK01/00870 and EP application No. 01610053.9 modified to provide a gradient. PCT/DK01/00870 and EP application No. 01610053.9 are hereby incorporated by reference.

The sheet-like substrate may comprise a polymer gradient coating on at least a part of the surface of the substrate, said coating representing a surface composition gradient in at least one direction along the surface of the substrate or the material.

The sheet-like substrate of a preferred embodiment could e.g. comprise a surface area, such as an area of between 0.1 and 100 cm², more preferably between 1 and 20 cm², wherein said part of the substrate surface, which preferably may be substantially plane, is coated with the polymer gradient coating. The polymer gradient coating should preferably constitute or represent a surface composition gradient essentially parallel to the plane of the coated surface of the substrate. The polymer gradient coating may preferably constitute a surface composition gradient in one direction essentially parallel to the plane of the surface of the substrate.

- The material according to the invention may e.g. comprise a substrate having one or more of the following gradients: One or more pH value gradients including gradients extending over at least 0.01 or 0.5 pH units, preferably at least 1 pH units, more preferably at least 2 pH units over the substrate surface and/or one or more hydrophilicity/hydrophobicity
- 5 gradient measured as surface tension, wherein the gradient extends over at least 1 dyne/cm, preferably at least 10 dynes/cm and more preferably at least 25 dynes/cm over the substrate surface, more preferably the gradient extension on the substrate is within 10-80 dynes/cm. The substrate may e.g. comprise several pH gradients and/or hydrophilicity/hydrophobicity gradient covering different ranges.
- 10 Generally, it is preferred that at least 20 %, preferably at least 40 % of the surface of the sheet-like substrate is covered with the polymer gradient coating. In many instances, such as those illustrated in the examples, the coating is present on essentially the entire surface area of at least one of the surfaces of the substrate.

- In a preferred embodiment, the sheet-like substrate is in the form of a tape or strip having
- 15 a thickness with an edge constituting a thickness surface, a first and a second side with a first and a second surfaces, respectively, at least one of said thickness surface and first and second surfaces being partly or totally covered with the polymer gradient coating. In this embodiment, it is preferred that at least one of said first and second surfaces is partly or totally covered with the polymer gradient coating, even more it is preferred that
- 20 essentially the whole of at least one of said first and second surfaces is covered with the polymer gradient coating. In this embodiment, it is most preferred that at least an elongated surface area of the tape or strip is coated, and that the coating constitutes or represents a surface composition gradient in one direction essentially parallel to the plane of the coated surface, where the direction preferably is essentially parallel to the longest
- 25 line between border lines of the elongated surface area. The elongated surface area may preferably be one of the major surfaces of the tape or strip.

- The sheet-like substrate may have any chemical composition such as polymers, e.g. polyolefins, such as polyethylene (PE) and polypropylene (PP) or other thermoplastics, such as polyester, polytetrafluoroethylene (PTFE), tetra-fluoroethylene-
- 30 hexafluoropropylene-copolymers (FEP), polyvinyl-difluoride (PVDF), polyamides (e.g. nylon-6.6 and nylon-11), and polyvinylchloride (PVC), rubbers e.g. silicon rubbers, glass, paper, aramide fibres, fibres, ceramics, metals, mixtures thereof etc. The material may further be made from or include any type of fibers and fiber reinforced materials. The

sheet-like substrate comprising the polymer gradient coating may preferably be porous, optionally with a porosity of at least 5 %, preferably at least 10 % by volume. The density of the material should preferably be below 0.9 g/cm^3 , more preferably below 0.5 g/cm^3 , in dry and non-compressed state. The substrate may e.g. be made from or include fibers or
5 foam. Preferably the substrate comprises pores or openings, which provide the substrate with a capillary effect to water.

Such materials may be in any suitable form, preferably representing a high surface area. The currently most interesting examples are porous and non-porous polyethylene, polyester or polypropylene felts, papers, non-woven glass fibres, etc. It is envisaged that
10 particularly interesting sheet-like substrates are polyethylene (EP), polypropylene (PP), or polyester or mixtures such as PE/PP felts. The material may include any type of fibers and fiber enforced materials. As will become apparent in view of the following, the sheet-like substrate will in the preferred embodiment constitute all or the main part of the material or sheet-like material. Thus, the mechanical properties (e.g. flexibility, strength,
15 etc.) of the material will to a great extent be determined by the selection of the sheet-like substrate. The substrate may preferably have a structure which allow the liquid comprising the biomolecules to pass through the substrate in a direction parallel to the gradient or gradients.

The substrate of the first group of preferred embodiments may be pre-coated in order to
20 modify the properties thereof, e.g. the ability of the surface to adhere to polymer gradient layer or the hydrophobic properties of the substrate as such. The pre-coating may preferably be in the form of a substantially uniform layer or it may alternatively be applied in a pattern such as a pattern providing a gradient as described in the following. Preferably, the pre-coating is in the form of a substantially homogenous layer of a polymer
25 onto the native surface of the substrate. The pre-coating may e.g. be performed in order to moderate the effect of the composition gradient. Thereby it may be possible to obtain an even higher resolution when separating organic compounds.

The polymer gradient coating of the preferred embodiments may in principle have any thickness, but as it will be clear to the skilled person a too thin layer or a too thick layer
30 may give a poor resolution and/or a prolonged separation time. Preferably the thickness of the polymer gradient layer is at least 5 nm, preferably in the range of up to about $100 \text{ }\mu\text{m}$, more preferably in the range of 10-1000 nm. In one embodiment the polymer gradient coating has a thickness of at the most 5000 nm. The thickness of the layer as such may of

course vary over the coated part of the surface of the substrate in accordance with the requirement for a composition gradient. Alternatively, and equally applicable, is a substantially uniform layer thickness, such as a thickness of the polymer gradient coating which is essentially the same through out the coating area.

- 5 In one preferred embodiment, the polymer gradient coating varies in thickness continuously or stepwise along its direction or directions of surface composition gradient. The polymer gradient coating preferably varies in thickness essentially continuously along its direction or directions of surface composition gradient.

- As described above, the polymer coating gradient of the first group of preferred
10 embodiments may be in the form of a polymer coating which varies in chemical composition so that it varies along the surface of the substrate in a graduating pattern, preferably so that the composition is varied in concentration of one or more components and/or in the form of a mixture of two or more compounds which is varied with respect to the amount of the respective compounds stepwise or continuously along the surface of
15 the substrate.

- The coating of the preferred embodiments provides a layer in the form of a polymer or polymeric composition as further described below, which does not have the same composition or concentration over the entire coated surface. The term "composition gradient" or "surface composition gradient" is intended to mean a variation of chemical
20 composition over a surface in a more or less gliding manner. The variation can be expressed as the variation in density of one or more chemical groups. The variance (gradient) may be steep or flat, or may even be step-wise so as to express a virtual gradient.

- The polymer gradient coating according to the first group of preferred embodiments
25 comprises one or more chemical components also designated monomers. The term monomers means components for the polymeric composition to provide the polymer gradient coating. The term monomers should in the present invention include any polymerisable component but it may also include components which are not polymerisable, i.e. do not covalently bind to the other component to thereby have high
30 molecular weight e.g. above 20,000. Thus the coating may include monomers which are not polymerised but are bound e.g. by an adhesive forces. The polymer gradient coating should preferably comprise or be made from at least 10 % by weight, more preferably at least 25 % by weight of polymerisable monomers. In one embodiment, the polymer

gradient coating consists essentially of polymerised monomers or is made from polymerisable monomers. Monomers which are not polymerised or polymerisable should preferably be fixed in or to the coating to avoid unintended migration of these components. As it will be clear to the skilled person, the components in the polymer

5 gradient coating may be any type of component, wherein preferably at least one of the components in the coating should, e.g. by ionic forces or van der Waals forces, attract at least one of the organic compounds in a mixture that is to be separated, such as proteins.

In an embodiment, the polymer gradient coating varies in composition continuously or step wise along its direction or directions of surface composition gradient, and the polymer

10 gradient coating varies in composition essentially continuously along its direction or directions of surface composition gradient, it is preferred that the gradient is composed from two or more monomers with varying amounts of the respective monomers in the polymer gradient composition along the direction or directions of surface composition gradient.

15 The polymer gradient coating may comprise acidic groups. In this embodiment, it is particularly preferred that the polymer gradient coating is made from monomers including monomers selected from the group consisting of acrylic acid, methacrylic acid and vinylacetic acid.

The polymer gradient coating according to the preferred embodiments may comprise

20 alkaline groups. In this embodiment, it is particularly preferred that the polymer gradient coating is made from monomers including monomers selected from the group consisting of cyanoacrylate, cyanomethacrylate, ethylene diamine and allylamine.

The polymer gradient coating according to the preferred embodiments may comprise neutral groups, such as pH neutral groups. In this embodiment, it is particularly preferred

25 that the polymer gradient coating is made from monomers including monomers selected from the group consisting of propylene, ethylene, styrene, methyl vinyl ether, hexene, ethyleneglycolvinylether, diethyleneglycolvinylether and vinylpyrrolidone.

In a particularly preferred embodiment according to the invention, the composition gradient is a pH gradient, and it is preferred that the polymer gradient coating is made

30 from monomers including at least mixtures of acrylic acid and cyanoacrylate, mixtures of acrylic acid and ethylene diamine, mixtures of acrylic acid and allylamine, or mixtures of vinylacetic acid and allylamine.

In another particularly preferred embodiment, the composition gradient is a hydrophobicity gradient, and in this embodiment it is preferred that the polymer gradient coating is made from monomers comprising one or more relatively hydrophobic monomers preferably selected from the group consisting of perfluorohexene, perfluoromethylpentene, hexene, pentene, cyclohexene, acetylene, styrene, vinylbornene, and mixtures thereof, and one or more relatively hydrophilic monomers preferably selected from the group consisting of vinylacetate, vinylpyrrolidone, ethyleneglycolvinylether, diethyleneglycolvinylether, methacrylate, methylmethacrylate, allyl alcohol and mixtures thereof.

The substrate may further comprise a top-coating, which e.g. may be a polymeric coating, such as a top-coating in the form of a polymer layer with a thickness of below 20 nm, preferably below 5 nm, and more preferably between 0.5 and 3 nm. This coating may be provided to protect the polymer gradient coating e.g. from CO₂, O₂ or any other gases that unintentionally may react or block the components in the polymer gradient coating, or the top-coating may modify or moderate the composition gradient of the material. In this way, the functional groups of e.g. a pH gradient (i.e. acidic and basic groups), may not bind so strongly to the molecules intended for separation and further high affinity non-specific bonding may be reduced.

The composition gradient can be prepared by plasma polymerisation of a coating gas onto a sheet-like substrate.

The material including the substrate used according to the first group of preferred embodiments may be prepared as described in PCT/DK01/00689. The surface gradient may be further refined by combining two plasma polymerised layers (two individual plasma polymerisation steps) having different properties in order to obtain the gradient surface. Illustrative examples of such "multi"-layered gradients are a) a gradient of an acidic monomer and a uniform layer of a basic monomer (or vice versa), b) a uniform layer of a basic monomer and a gradient of an acidic monomer (or vice versa); c) a gradient of an acidic monomer and counter-directional gradient of a basic monomer.

Alternative coating process

Examples of such general deposition/polymerisation processes are described in the recent patent applications by Creavis, see e.g. EP 1 018 531 A2, WO 00/44818, EP 1 040 874 A2, which are hereby incorporated by reference. The deposition of monomers should of course be controlled by suitable means in order to provide the composition gradient.

The coating process includes vaporisation coating wherein the steps of applying the monomers onto the surface of the substrate; and bringing the monomers to polymerises; are carried out by evaporation of the monomers, deposition of the monomers and optionally a catalyst onto the substrate to provide a gradient, and bringing the monomers
5 to react by free radical polymerisation of the monomers to form the polymer coating in such a manner that the composition gradient is prepared.

The monomer should basically be vaporisable for this process. The catalyst may in principle be any type of catalyst useful for providing a sufficient polymerisation. The catalyst may be applied in a separate step or simultaneously with the other components.
10 The composition gradient may be provided by use of masking as described above under the plasma process.

Providing a liquid comprising the biocomponents

The biocomponents to be contacted with the separation layer, i.e. in the form of a gradient surface of the sheet-like substrate should be present in a suitable liquid so as to facilitate
15 the distribution of the biocomponents onto the substrate. Thus, the biocomponents will be prepared as a sample either dissolved or dispersed in a liquid. The liquid may be of the type normally used as working liquids in gel separations and other handling of biocomponents. Liquids for such use are generally known in the art, and the skilled person will by use of his general common knowledge be able to select a suitable liquid for
20 the respective biocomponents or combinations of biocomponents. Water, mixtures of water, salts and/or organic constituents e.g. water miscible organic solvents are normally used for this purpose. The biocomponents may also be dispersed or dissolved in human liquid, such as serum.

The actual process of preparing the sample varies from sample type to sample type, i.e.
25 according to the source and properties of the biocomponents. The different sample preparation processes do not only differ depending upon the type of source and biocomponents, but also with respect to the subset of biomolecules (e.g. protein/protein complex) it is desirable to separate and/or isolate. Obviously, the sample preparation will be adjusted according to parameters known to the person skilled in the art.

30 The liquid should thus preferably be a solvent or a dispersion of the biocomponents such as an organic or an aqueous solvent. In most situations it is preferred that the liquid comprises at least 25 % by vol. of water, more preferably comprising at least about 45 %

- by vol. of water. The liquid or solvent may further comprise other components such as acetic acid, ethanol, glycerol, detergents such as CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (detergent)) and SDS (Sodium Dodecyl Sulphate (charged detergent)) and buffer systems e.g. comprising one or more components e.g.
- 5 including chaotropic agents, such as for example of the following components: β -mercaptoethanol, urea, thiourea, guanidinium chloride and DTT)

One example of a simple preparation methodology useful where the source of the biocomponents (here: proteins/protein complexes) is cells from a culture is simply to remove the culture medium that the cells have been growing in and add a "lysis buffer"

10 (e.g. about 7 M urea, about 2 M thiourea, about 2% CHAPS about 0.5% DTT, about 2% pharmalytes).

Two other applicable types of buffers are: (a) about 50% ethanol, about 1% acetic acid about 49% water (as an organic solvent) which is particularly useful for hydrophobic proteins; and (b) about 10% glycerol, about 2% SDS, about 60 mM Tris·HCl pH 6.8, about

15 5% β -mercaptoethanol (as the classical sample buffer for one dimensional separation of proteins in a gel) which is particularly useful for larger proteins (and to some extent also for hydrophobic proteins).

The above three buffers may cover a broad range of biocomponents, but alternatives and modifications will be recognisable for the person skilled in the art.

- 20 The biocomponents are typically present in the liquid as a mixture of numerous individual types of biocomponents. The process of the invention is intended for the isolation of all or just a selection of those biocomponents and/or for the spatial separation of the individual biocomponents on the layer/gradient surface of the sheet-like substrate. The separation is essentially independent of the relative concentrations of the biocomponents in the liquid.
- 25 The sample to be separated may contain between 2 and 150.00 biocomponents or even more. Dependent on the type and combination of biocomponents it may be possible to obtain a separation of 5,000, 10,000, 100,000, 150,000 or even more different biocomponents.

The liquid containing the biocomponents may in principle contain as many biocomponents

30 as possible, provided that the biocomponents are not dried. Generally used biocomponent concentrations are between 1-20 $\mu\text{g}/\mu\text{l}$, such as between 5 and 10 $\mu\text{g}/\mu\text{l}$. In case of

proteins or protein complexes the concentration may preferably be between 7-9 $\mu\text{g}/\mu\text{l}$, whereas in case of DNA the concentration could be between 9-11 $\mu\text{g}/\mu\text{l}$. During the step of separation the concentration will be reduced. In order to obtain an optimal resolution the concentration of the biocomponent may preferably be even less than indicated above, 5 e.g. between 0.1 and 5 $\mu\text{g}/\mu\text{l}$, such as about 2, 3 or 4 $\mu\text{g}/\mu\text{l}$.

The biocomponent may preferably be labelled such as it is generally known to label biocomponents such as biomolecules e.g. proteins. The labelling may e.g. include radioactively labelling, fluorescence labelling and other e.g. chemicals with various groups which could act as handles or functional groups for subsequent processes.

- 10 Further information concerning the method of preparing the biocomponents and the liquid with the biocomponents may be found in US 5264101, which is hereby incorporated by reference.

Contacting the liquid with the surface separation layer

- The liquid comprising the biocomponents - or rather a mixture of biocomponents - may 15 preferably be contacted with the surface separation layer by applying the liquid to the substrate with the surface separation layer e.g. for a period sufficiently long for the biocomponents to become adsorbed to the substrate and/or the surface separation layer. The liquid may be applied directly to the surface separation layer on the substrate or it may be applied to the substrate which transfer it to the surface separation layer. In the 20 following the term "substrate" also include the surface separation layer unless other is mentioned.

The time necessary for the biocomponents to become absorbed to the substrate is given by thermodynamic parameters, but will mostly depend on how the liquid is distributed on or applied to the substrate and the nature of the substrate and the liquid (e.g. viscosity).

- 25 The liquid may be loaded onto the substrate using "cup loading", i.e. applying the liquid to one of the substrate ends or to an area e.g. a local area anywhere along the length of the substrate. If the substrate comprises a pH gradient, it is preferred that the liquid is at the most neutral point, e.g. dripped down onto the mid part of the substrate. Most preferably the liquid is added where the pH value are in the interval 5-7, if this pH value or values are 30 included in the gradient.

The liquid may alternatively be distributed over parts or all of the substrate, such as distributed evenly over the surface of the substrate.

The substrate may e.g. be wetted with a liquid prior to the application of the liquid containing the biocomponent. This aspect is particular relevant if the substrate is capable
5 of absorbing or swelling liquid as this absorption or swelling may provide conductivity through the substrate and may decrease the necessary amount of sample.

In one embodiment, the liquid comprising the biocomponents is desalted prior to the loading. This may be carried out e.g. by electrophoresis or chromatography, such as affinity chromatography. However, any other means may also be used.

10 The material may be mounted on a carrier before being brought into contact with the liquid. The carrier may in principle be of any material and type, and have any shape e.g. curved or plane. The carrier may preferably be of a non-absorbing material e.g. in the form of a glass plate. Several substrates or materials e.g. two or more may be mounted to one carrier. Generally it is preferred that the material are of a material that does not retain
15 the biocomponents from entering the substrate.

In one instance, the sheet-like material is mounted on the surface of plane or curved plate. The liquid is applied in one end and the plate is kept at an angle to thereby distribute the liquid.

In another instance, the sheet-like material is mounted on the surface of plane or in
20 particular the outer curve of a curved plate. The plate with the substrate or at least the side of the plate with the substrate is dipped into the liquid.

In both instances, it is possible to distribute a relatively small volume of liquid quite evenly over the surface of the substrate.

It should be understood that it is not necessary that all of the biocomponents of the
25 mixture are adsorbed to the substrate; some may be more favourably distributed in the liquid phase, e.g. due to the hydrophobic/hydrophilic conditions.

It should also be understood that a plurality of sheet-like substrates may be coiled (or otherwise mounted) on the same carrier. This is particularly relevant where the plurality of sheet-like substrates represent overlapping gradients, e.g. pH gradients. In this way, a

plurality of relatively short sheet-like substrates each covering a pH range of e.g. 1 pH unit (or e.g. 0.5 pH unit) may be used to cover a broader pH range.

After the liquid has been brought into contact with the substrate for a sufficient period of time, non-adsorbed biocomponents and liquid may be removed.

- 5 The above steps may be sufficient to obtain a suitable separation (or isolation) of biocomponents of interest due to the fact that the biocomponents will have a preference for adsorption to an area of the gradient surface which corresponds to the hydrophilicity/hydrophobicity/pH/etc. of the biocomponent.

- In situation where the gradient is a structure gradient the position of the substrate may be
10 important i.e. if the gradient is a graduating number of cross-links, the end of the substrate comprising the surface gradient having the lowest number of cross-links should preferably be kept higher than the end of the substrate comprising the surface gradient having a higher number of cross-links, in particular if a voltage over the gradient is not applied. If the gradient is a graduating number of cross-links, the liquid should preferably be applied
15 at the end of the gradient where the number of cross-links is lower.

Electrophoresis

Normal electrophoresis techniques may be applied in order to refine the resolution of the biocomponents, such as biomolecules (e.g. proteins). This constitutes a particularly preferred embodiment of the present invention.

- 20 As an example, a voltage over a stretch or length of the sheet-like substrate in a direction substantially parallel to the composition gradient may be applied, preferably via clamps near the longitudinal ends of the material. The equipment for applying a voltage over the material may be similar to that used for gel electrophoresis. A voltage of up to 50,000 V/m or even more. Typically, up to about 20,000, 10,000, 5,000 or 3,000 V/m may be
25 applicable to separate biocomponents, such as proteins. The longer the sheet-like material, the longer run times will be needed, thus, for materials having a length in the order of meters, a run time in the order of hours or even days may be necessary, however when using a plurality of shorter "strips" which all in all cover the relevant gradient e.g. pH, hydrophobicity, etc. range, the operation time may be reduced considerably.
- 30 Illustrative conditions for application of a voltage over a sheet-like material can be similar to those described in the manual for the commercially available "Multiphore" product

("Multiphor II Electrophoresis System" from Amersham Pharmacia Biotech AB). The voltage may e.g. be applied to the substrate using electrode wicks e.g. IEF electrode wicks from Amersham Pharmacia Biotech AB.

The running conditions can of course be further optimised with due consideration of the
5 gradient properties of the sheet-like materials (strips). The actual optimal voltage gradients and total values can be broadly varied. Currently, a voltage of up to 3,500V has proven beneficial. A total Volt*hours may be from 20 V*hours to 5000 KV*hours, such as between 45 V*hours and 1000 KV*hours, between 100V*hours and 500 KV*hours or between 225 V*hours and 100 KV*hours.

- 10 The voltage may preferably be applied in a direction substantially parallel to a gradient. The voltage may be applied over the total length of the substrate parallel to the composition gradient, or over parts of the gradients.

In one embodiment, the air above the substrate is kept free of oxygen or CO₂ e.g. by blowing with nitrogen. The presence of oxygen or CO₂ may preferably be avoided since
15 these may react with the substrate, the liquid or the biocomponents. By blowing nitrogen, a cooling of the substrate may also be achieved.

The substrates or strips are often temperature regulated, e.g., to a temperature between 5-60 °C, such as about 20°C. This may be carried out using any method e.g. by placing them on a plate through which water is circulated at the desired temperature. Other
20 desired methods include applying a carrier with the substrate or the substrate directly, particularly if the substrate is non-absorbing onto a cooling plate. The cooling should be performed without evaporating too much liquid from the sample, and generally if blow-cooling is used, the gas or air should have a high moisture level such as above 80 % of saturation. To avoid extensive evaporation of the liquid of the sample the substrate or the
25 carrier with the substrate may be placed in a closed or partly closed chamber with a 'humidification' water bath.

The application of voltage may result in an increased heat generation, in particular if the voltage is increased quickly. In order to optimise and regulate the cooling the voltage may preferably be raised stepwise or continuously over a period of from 5 minutes to 2 or 3
30 hours. This may also result in a desalting of the liquid, which may further reduce the generation of heat, in particular for samples containing components which could form urea such a basic proteins due to avoidance of break down of urea into cyanate ions which

occurs at high temperature or highly basic conditions or both. These features may be significant for obtaining a far better focussing and a substantial improvement of the reproducibility of the quantitative data.

Generally it is preferred that the voltage is applied being applied in at least a first and a
5 second step, wherein the voltage in the first step is substantially lower than the voltage in the second step, preferably the voltage in the first step is at least 100 V/m or even at least 500 V/m lower than the voltage in the second step.

In situations where the voltage applied is applied in at least a first and a second step, the voltage in the first step may be raised continuously or by small steps from 0 to between
10 300 and 4000 V/m, preferably between 1000 and 2500 V/m. The increase continuously or stepwise in voltage in the first step e.g. using small steps may preferably be less than 60 V/minute, preferably less than 30 V/minute, more preferably between 10 and 15 V/minute. The term "small step" includes steps of up to about 50 V/m.

In situations where the voltage applied is applied in at least a first and a second step, the
15 voltage in the second step may be raised continuously or stepwise up to about 50,000 V/m, preferably up to about 20,000 V/m, such as 10,000, 5,000 or 3,000 V/m, the increase in voltage in the second step preferably being between 30 and 1000 V/minute.

When applying the voltage in at least a first and a second step, the voltage in the first step may preferably be raised continuously and the voltage in the second step may preferably
20 be raised faster than the voltage being raised in the first step.

As an illustrative example of a procedure for applying the voltage may be as follows:

1. The substrate e.g. with a 250 mm gradient or the carrier with the substrate is preferably placed on a constant-temperature surface.
2. Electrode wicks and electrodes are positioned at the ends of the substrate.
- 25 3. A power pack is programmed to give a linear gradient, for example from 0 to 600V in 2 hrs, followed by a ramp up to about 4000 V in the following 1-5 hrs e.g. about 2-4 hours. Finally the power pack is run at maximum power until the electrophoresis is complete, e.g. up to 18 hours.

Particularly long sheet-like materials e.g. longer than 30 cm, 50, cm, 100 cm or even longer, may be cut in smaller pieces (either before or after application of the liquid) and a voltage may be applied over the materials either individually or simultaneously.

Analysis

- 5 After having separated the biocomponents the biocomponents may be directly identified on the surface and or a quantitative analysis or further separation or identification may be performed. A quantitative analysis may be performed using standard methods, such as chemical methods, fluorescence, or determination of radioactivity.

In general the biocomponents may be analysed by similar methods as those used for gel
10 electrophoresis, e.g. by mass spectrometry, further separation, etc.

When further separated by gel electrophoresis, e.g. on a conventional gel, the biocomponents adsorbed to the strip are loaded onto the gel by placing the strip on top of the gel and then sealing it in place, e.g. with a 1% agarose solution dissolved in a SDS buffer.

- 15 When applying a step of mass spectrometry, a procedure like the following can be used:
- 1) the process according to the invention is conducted;
 - 2) the strip is passed through a devise in order to determine the protein concentration (this could be a fluorescent camera or a scintillation counter with a slit window);
 - 3) the strip is (optionally) treated with enzymes to digest the proteins;
 - 20 4) the strip is infused with a laser absorbing dye; and
 - 5) the strip is "run" in the mass spectrometer and "read" like a video tape.

The procedure can be conducted automatically and can be controlled by computer. Such a computer program can be linked to the Internet or to local downloaded non-redundant databases of DNA, RNA (ESTs) and protein sequences).

- 25 By use of the invention an improved biocomponent (protein) resolution has been provided. It has further been made possible to use solvents or liquids which are generally not useful for electrofocussing gel electrophoresis (where solvents for gels are typically limited to hydrophilic non-ionic detergent containing solvents). It is believed that this will allow for a wider range of proteins to be resolved than by gels (e.g. membrane proteins, very acidic
30 proteins (below pH 4.0) or basic proteins (above pH 9.0), very low molecular weight

proteins (below 5,000 Da) or very high molecular weight proteins (above 250,000). For this embodiment it has further been made possible to reduce the scale of the strips to nanoscale technologies because the separation occurs essentially on a surface.

DESCRIPTION OF FIGURE 1

5 The figure illustrates a plasma reaction chamber 1, which is useful in the process according to the invention. The reaction chamber 1, also designated a vacuum chamber, comprise a winding system 2 in the form of two reels onto which a sheet-like substrate in the form of a tape or similar can be wound during the application of a coating. The system comprises a number of supply units 4 comprising the monomers e.g. as indicated
10 in the form of acid, basic, and neutral components. Each supply unit comprises a flow control 3 for controlling the respective amount of monomer added to the reaction chamber. The reaction chamber 1 comprises two electrodes, 6, 7 which may be of stainless steel. In the shown reaction chamber one of the electrodes 6 is constituted by the outer wall and the other electrode is constituted by grid placed in a distance from the
15 wall. An isolating material 5 e.g. of PE is placed around the electrode wall 6. A substrate 8 is wound from one of the reels to the other during the coating. The plasma 9 is generated in the reaction chamber, and monomer as well as inert gasses such as Ar is added as indicated by the arrows 10, 11.

EXAMPLES

20 **Manufacture of materials**

Example 1 - Manufacturing a pH-gradient for separation of proteins.

The substrate for the pH-gradient was a polyethylene/polypropylene (PE/PP) felt from Freudenberg (VK1099, 60 g/m²), which was available in 30 mm wide rolls.

The substrate felt was spooled from one reel to another under the plasma polymerisation.
25 Hereby the felt passed through the plasma reaction chamber; about 16 cm of the felt (in the length-wise direction) was exposed in the reaction chamber. The plasma was generated by a 2-phase AC supply.

The plasma polymerisation of the acidic and basic monomers took place in the reaction chamber by simultaneously varying the mixture of acidic and basic monomers. Acrylic
30 acid was used as the monomer having an acidic group and allylamine was used as

monomer having a basic group. The acrylic acid was bubbled through with argon. An additional Argon inlet ("add Ar") to the reaction chamber was used to regulate the partial pressure of the monomers in the chamber.

The reels for spooling were placed in boxes provided with a flow of argon (slight over
5 pressure in relation to the polymerisation area) to prevent undesirable downstream polymerisation ("protection Ar").

The reaction chamber with reels was essentially constructed as outlined in Fig. 1.

Procedure:

The felt was placed on the winding-equipment (reels). The vacuum chamber was closed
10 and evacuated to a pressure of 10 μ bar. A flow of argon for bubbling the acrylic acid was adjusted to 20 sccm (standard cubic centimeters per minute (ml/min)) with the use of a flow controller. The flow of argon/acrylic acid was adjusted to 25 sccm. The flow of allylamine was adjusted to 3 sccm. The flow of "add Ar" was adjusted to 35 sccm. The flow of "protection Ar" was adjusted to 10 sccm. The pressure was increased to 140 μ bar
15 and was kept constant at this level during the plasma polymerisation.

The plasma was turned on with an effect of 2.5 W/l and was kept constant at this level during the plasma polymerisation. Simultaneously the spooling of the felt was started with a speed of 4 cm/min corresponding to a "residence time" in the plasma reaction chamber of 4 min.

20 4 min. after the start of the spooling, a computer-controlled change of the acrylic acid and allylamine flows started. The total flow of monomer (combined molar flow of acrylic acid and allylamine) was almost constant. After 14 min of plasma polymerisation (corresponding to 0.4 metres of felt) the flow of argon/acrylic acid was 40 sccm, the flow of allylamine was 1.5 sccm.

25 The plasma was turned off, and the spooling was stopped. All flows, except for that of "protection Ar", were interrupted. The pressure was adjusted to 20 μ bar. After 1 min at this pressure, the flow of "protection Ar" was also interrupted. The pressure was raised to atmospheric pressure and the vacuum chamber was opened. The felt could be removed from the reels.

The reaction provided a felt having a pH gradient on the surface thereof. By test with a pH-liquid, the strips showed a pH-range from 5 to 5.5 which equals 1 pH-unit per 0.8 metre. The thickness of the plasma polymerised layer of acidic and basic monomer was in the range of 30-40 nm. For protein separation, the felt was cut into strips of 3 mm width, 5 spooled and packed.

Example 2 - Manufacturing a pH-gradient for separation of proteins in 2 steps.

The substrate for the pH-gradient was a polyethylene/polypropylene (PE/PP) felt from Freudenberg (VK1099, 60 g/m²), which was available in 30 mm wide rolls.

The substrate felt was spooled from one reel to another under the plasma polymerisation. 10 Hereby the felt passed through the plasma reaction chamber; about 16 cm of the felt (in the length-wise direction) was exposed in the reaction chamber. The plasma was generated by a 2-phase AC supply.

The plasma polymerisation of the acidic and basic monomers took place in 2 steps:

- 1) Polymerisation of the basic monomer in a homogeneous layer
- 15 2) Polymerisation of the acidic monomer layer with varying thickness.

Acrylic acid was used as the monomer having an acidic group, and ethylenediamine was used as monomer having basic groups. Both monomers were bubbled through with argon.

The reels for spooling were placed in boxes provided with a flow of argon (slight over 20 pressure in relation to the polymerisation area) to prevent undesirable downstream polymerisation ("protection Ar").

The reaction chamber with reels was essentially constructed as outlined in Fig. 1.

Procedure:

The felt was placed on the winding-equipment (reels). The vacuum chamber was closed 25 and evacuated to a pressure of 10 µbar.

Step 1)

The flow of argon for bubbling the ethylenediamine was adjusted to 30 sccm. (standard cubic centimeters per minute (ml/min)) with the use of a flow controller. The flow of argon/ethylenediamine was adjusted to 60 sccm.

The flow of "protection Ar" was adjusted to 10 sccm. The pressure was increased to 150
5 μ bar and was kept constant at this level during the plasma polymerisation in step 1).

The plasma was turned on with an effect of 7 W/l and was kept constant at this level during the plasma polymerisation. Simultaneously the spooling of the felt was started with a speed of 4 cm/min corresponding to a "residence time" in the plasma reaction chamber of 4 min.

10 After 14 min the plasma was turned off, and the spooling was stopped. All flows, except for that of "protection Ar", were interrupted. The pressure was adjusted to 10 μ bar.

Step 2)

After "rewinding" the felt, the flow of argon for bubbling the acrylic acid was adjusted to 20 sccm. The flow of argon/acrylic acid was adjusted to 40 sccm.

15 The pressure was increased to 140 μ bar and was kept constant at this level during the plasma polymerisation in step 2).

The plasma was turned on with an effect of 2.5 W/l and was kept constant at this level during the plasma polymerisation. Simultaneously the spooling of the felt was started with a speed of 4 cm/min corresponding to a "residence time" in the plasma reaction chamber
20 of 4 min.

After 4 min. after the start of the spooling, a computer-controlled change of the acrylic acid started. After 14 min of plasma polymerisation (corresponding to 0.4 metres of felt) the flow of argon/acrylic acid was 25 sccm.

The plasma was turned off, and the spooling was stopped. All flows, except for that of
25 "protection Ar", were interrupted. The pressure was adjusted to 20 μ bar. After 1 min at this pressure, the flow of "protection Ar" was also interrupted. The pressure was raised to atmospheric pressure and the vacuum chamber was opened. The felt could be removed from the reels.

The reaction provided a felt having a pH gradient on the surface thereof. By test with a pH-liquid, the strips showed a pH-range from 6.5 to 7.5 which equals to 1 pH-unit per 0.4 metre. For protein separation, the felt was cut into strips of 3 mm width, spooled and packed.

5 *Example 3 - Modification of pH gradient*

The felt provided with a pH gradient as described in Example 1 or 2 was modified in order to make the functional groups of the plasma polymerised coating less susceptible to reaction with the organic molecules to be separated on the surface. This was done by providing a thin layer of a pH inert polymer on top of the polymers representing the pH
10 gradient. This modification procedure followed the above process after the plasma was turned off (i.e. before the "All flows, except for that of "protection Ar", were interrupted"):

All flows, except for "protection Ar", were interrupted. After 2 min, a flow of diethylenglycolvinylether was applied; flow rate 20 sccm. After another 2 min, the plasma was turned on with an effect of 4 W/l and the spooling was started as "rewinding" with a speed
15 of 20 cm/min. After coating the length of the pH gradient, the plasma was turned off, the diethylenglycolvinylether flow was interrupted and the pressure was lowered to 20 μ bar. After 1 min at this pressure, the flow of "protection Ar" was interrupted and the pressure was allowed to raised to atmospheric pressure and the vacuum chamber was opened. The felt could be removed from the reels.

20 The thickness of the polymerised diethylenglycolvinylether was around 3-4 nm. By test with a pH-liquid, the strips showed a pH-range comparable with the uncoated strips.

After the plasma polymerisation the roll could be sliced into strips of 3 mm width for the protein separation.

EXAMPLE 4 – SEPARATION OF BIOMOLECULES BY ELECTROPHORESIS

25 The felt provided with a pH gradient as described in example 1-3 or by other means was used for separation of proteins in an electrophoresis based process with a maximum voltage of 1250 V. The proteins were radioactive labeled in order to detect and evaluate the separation process.

Procedure

- 1) Strips were cut from the felt with a gradient produced as described in example 1. The strips had a length of 250 mm and a width of 3 mm. The strips were placed on the cooling plate of a Multiphor II from Amersham Pharmacia Biotech AB.
- 5 2) 130 μ l of IPG A Lysis buffer was equally applied to the surface of each strip. 20 μ l sample of protein consisting of 100 μ g non labeled HeLa-cells and HeLa-cells radioactively labeled with [35 S]-Methionine 2 million CPM in an IPG A Lysis buffer were acidic cuploaded.
- 3) 10 mm wide electrode wicks (IEF Electrode Strip (18-1004-40) from Amersham
10 Pharmacia Biotech AB) saturated with water were placed on each end of the strips, such that the strip end was just covered. The positive electrode from the Multiphor was placed at the centerpart of the electrode wick in the acid end of the strip and the negative electrode from the Multiphor was placed at the centerpart of the electrode wick in the basic end of the strip. The plastic lid of the Multiphor was put in place.
- 15 4) Voltage was applied to the system from a programmable Power Supply (EPS 3501 XL from Pharmacia Biotech AB). Following the following linear ramp steps:

0 to 600 V in 135 minutes

600 to 1250 V in 60 minutes

Then the voltage was held constant at 1250 V for additionally 120 minutes and then the
20 electrophoresis was terminated.

During the electrophoresis the current in the system is surveyed in order to continuously monitor the process.
- 5) The strips were removed from the Multiphor, and the distribution of proteins were examined by exposure to x-ray sensitive films. The amount of proteins in the electrode
25 wicks was determined by radioactive counting.

The separation of protein is evident from a number of bands indicating high concentration of focused proteins visible on the x-ray film. Test in 2nd dimension electrophoresis - where proteins are separated due to molecular weight - show that the bands are in fact specific proteins.

The voltage and current profile is shown in figure 2.

EXAMPLE 5 – SEPARATION OF BIOMOLECULES BY ELECTROPHORESIS

The felt provided with a pH gradient as described in example 1-3 or by other means was
5 used for separation of proteins in an electrophoresis based process with a maximum
voltage of 3500 V. The proteins were radioactively labelled in order to detect and evaluate
the separation process. During the electrophoresis the strips are in an inert atmosphere.

Procedure

1) Strips were cut from the felt with a gradient produced as described in example 1. The
10 strips had a length of 250 mm and a width of 3 mm. The strips were placed on the cooling
plate of a Multiphor II from Amersham Pharmacia Biotech AB.

2) 130 μ l of IPG A Lysis buffer was equally applied to the surface of each strip. 20 μ l
sample of protein consisting of 100 μ g non labeled HeLa-cells and HeLa-cells
radioactively labeled with [35 S]-Methionine (2 million CPM) in an IPG A Lysis buffer were
15 acidic cuploaded.

3) 10 mm wide electrode wicks (IEF Electrode Strip (18-1004-40) from Amersham
Pharmacia Biotech AB) saturated with water were placed in each end of the strips, such
that the strip end was just covered. The positive electrode from the Multiphor was placed
at the centerpart of the electrode wick in the acid end of the strip and the negative
20 electrode from the Multiphor was placed at the centerpart of the electrode wick in the
basic end of the strip. The plastic lid of the Multiphor was put in place.

4) Nitrogen was by tubing led to the Multiphor, where it leads the nitrogen in under the
cooling plate in order to keep an oxygen and CO₂ free atmosphere. The nitrogen flow
shall be high enough to ensure an oxygen free atmosphere, but not so high that the strips
25 are dried out. The used flow rate was 80 ml pr. minute.

5) Voltage was applied to the system from a programmable Power Supply (EPS 3501 XL
from Pharmacia Biotech AB). Following the following linear ramp steps:

0 to 600 V in 135 minutes

600 to 3500 V in 60 minutes

Then the voltage was held constant at 3500 V for additionally 1200 minutes and then the electrophoresis was terminated.

During the electrophoresis the current in the system is surveyed in order to continuously
5 monitor the process.

6) The strips were removed from the Multiphor, dried and the distribution of proteins were examined by exposure to x-ray sensitive films. The amount of proteins in the electrode wicks is determined by radioactive counting.

The separation of protein is evident from a number of bands indicating high concentration
10 of focused proteins visible on the x-ray film. The test in 2nd dimension electrophoresis - where proteins are separated due to molecular weight - shows that the bands are in fact specific proteins.

The voltage and current profile is shown in figure 3.

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Claims:

1. A process for the separation of biocomponents

comprising the steps of:

- providing a material comprising a substrate which has a surface separation layer on at
5 least a part of its surface;
- providing a liquid comprising the biocomponents; and
- contacting the liquid comprising the biocomponents with the surface separation layer.

2. A process according to claim 1, wherein the biocomponent includes one or more of the
components selected from the group consisting of tissue, cells, body fluids, blood
10 components, microorganism, derivatives thereof, or parts thereof.

3. A process according to any one of the claims 1 and 2, wherein the biocomponent
includes one or more biomolecules of microbial, plant, animal or human origin or synthetic
molecules resembling them, preferably selected from the group consisting of proteins,
nucleic acids, such as RNA, DNA, PNA, oligonucleotides, peptides, hormones, antigens,
15 antibodies, and complexes including one or more of these molecules, said biomolecule
preferably being selected from the group consisting of proteins and protein complexes.

4. A process according to any one of the claims 1, 2 and 3 wherein the substrate is of one
or more materials, such as layered materials, said one or more materials being selected
from the group consisting of glass, glass-fiber based materials, metals, solid or foamed
20 polymers, non-woven or woven polymers, paper, fibres, such as carbon fibres; aramide
fibres; fiberreinforced materials; ceramics; or mixtures or combinations thereof.

5. A process according to any one of the preceding claims wherein the substrate is of one
or more materials including one or more polymer, said one or more polymer preferably
being in the form of non-woven polymer fibers, and the polymer preferably being selected
25 from the group consisting of polyolefins including polyethylene (PE) and polypropylene
(PP); polyester; polytetrafluoroethylene (PTFE); tetra-fluoroethylene-hexafluoropropylene-
copolymers (FEP); polyvinyl-difluoride (PVDF); polyamides; polyvinylchloride (PVC),
rubbers such as silicon rubbers and mixtures thereof.

6. A process according to any one of the preceding claims wherein the surface separation layer comprises a gradient, defined as a stepwise or continuous gradient in the layer composition or structure in at least one direction along the surface of the substrate.
7. A process according to claim 6 wherein the surface separation layer comprises a
5 structure gradient, said structure gradient being in the form of a stepwise or continuously graduating structure change selected from the group consisting of change in thickness, change in cross-linking degree, change in density and change in molecular weight.
8. A process according to any one of the claims 6 and 7 wherein the surface separation
10 layer comprises a composition gradient in the form of a stepwise or continuously graduating composition change selected from the group consisting of change of the concentration of one, two or more components, change of type of components so that at least one property, such as hydrophilicity or pH value is changing continuously or stepwise.
9. A process according to any one of the preceding claims wherein the substrate is coated
15 with a coating on at least a part of the surface of the substrate, said coating representing a surface composition gradient in at least one direction along the surface of the substrate, said coating preferably being a polymeric coating.
10. A process according to any of the preceding claims, wherein the surface separation
20 layer comprises a composition gradient, said composition gradient being a pH gradient or a hydrophobicity gradient.
11. A process according to any of the preceding claims, wherein the surface separation layer comprises a composition gradient, the composition includes a ligand with a functional group, which functional group can bind to one or more of the biocomponents to be separated, the gradient is constituted by a change of the number of ligands.
- 25 12. A process according to claim 11, wherein the ligand is linked to the surface through a photochemically reactive group, such as a quinone e.g. an anthraquinone.
13. A process according to any of the preceding claims, wherein the surface separation layer includes one or more of the components selected from the group consisting of acids, such as organic acids, amino acids, lipid acids and poly acids thereof; bases such
30 as organic bases, amino acids and poly bases thereof; aromates; metal components, such a organometals; halogens; zwitter ions e.g. ampholines; antigens and antibodies..

14. A process according to any one of the preceding claims wherein the gradient is in a direction substantially parallel to the longitudinal axis of the sheet-like substrate.
15. A process according to any one of the preceding claims wherein said material essentially is constituted of said substrate.
- 5 16. A process according to any one of the preceding claims wherein said material further comprises a supporting element for the substrate, the total material preferably being essentially sheet-like, more preferably said supporting element being a supporting sheet selected from the group consisting of polymers, such as polyolefins including polyethylene (PE) and polypropylene (PP); polyester; polytetrafluoroethylene (PTFE); tetra-
10 fluoroethylene-hexafluoropropylene-copolymers (FEP); polyvinyl-difluoride (PVDF); polyamides; polyvinylchloride (PVC); rubbers such as silicon rubbers; glass; paper; fibres, such as carbon fibres; aramide fibres; fiberreinforced materials; ceramics; metals or mixtures or combinations thereof.
17. A process according to any one of the preceding claims wherein said substrate has a
15 length parallel to the gradient which is at least 100 mm, preferably at least 200 mm, more preferably at least 500 mm, and even more preferably at least 1,000 mm.
18. A process according to any one of the claims the preceding claims wherein said gradient coating varies in composition or structure continuously or stepwise along its direction or directions of surface composition gradient(s), said polymer gradient coating
20 preferably varying in composition essentially continuously along its direction or directions of surface composition gradient (s), said composition preferably being composed of two or more monomers with varying amounts of the respective monomers in the gradient composition along the direction or directions of surface composition gradient.
19. A process according to any one of the preceding claims wherein said liquid is a
25 solvent or a dispersion of the biocomponents, said solvent preferably being an organic or an aqueous solvent, preferably comprising at least 25 % by vol. of water, more preferably comprising at least about 45 % by vol. of water.
20. A process according to any one of the preceding claims wherein said liquid is a solvent or a dispersion of the biomolecules, said solvent comprising one or more of the
30 components selected from the group consisting of acetic acid, ethanol, glycerol, phenol, detergents, and buffer systems such as a buffer system comprising one or more of the

components selected from the group consisting of β -mercaptoethanol, urea, thiourea, guanidinium chloride and DTT).

21. A process according to any one of the preceding claims wherein said material is mounted on a carrier before being brought into contact with the liquid, said carrier
5 preferably being a non-adsorbing plate, such as a glass plate.
22. A process according to any of the preceding claims, wherein two or more sheet-like substrates or materials are used in the separation, said sheet-like substrates or materials preferably being mounted to one carrier.
23. A process according to any one of the preceding claims wherein the liquid comprising
10 the biocomponents is loaded onto the substrate using cuploading, said substrate optionally being wetted prior to the cuploading.
24. A process according to any one of the preceding claims wherein the liquid comprising the biocomponents is desalted prior to the loading.
25. A process according to any one of the preceding claims further comprising the step of
15 applying a voltage over a stretch of the sheet-like substrate in a direction substantially parallel to the gradient, said voltage preferably being applied over the total length of the substrate parallel to the composition gradient.
26. A process according to claim 25 wherein the voltage applied is applied in at least a first and a second step, the voltage in the first step being lower than the voltage in the
20 second step, preferably the voltage in the first step being substantially lower than the voltage in the second step.
27. A process according to claim 25 or 26 wherein the voltage applied is applied in at least a first and a second step, the voltage in the first step being raised continuously from 0 to between 300 and 4000 V/m, preferably between 1000 and 2500 V/m, the increase in
25 voltage in the first step preferably being less than 60 V/minute, preferably less than 30 V/minute, more preferably between 10 and 15 V/minute.
28. A process according to any one of the claims 25, 26 and 27 wherein the voltage applied is applied in at least a first and a second step, the voltage in the second step being raised continuously up to about 50,000 V/m, preferably up to about 20,000 V/m,

such as 10,000, 5,000 or 3,000 V/m, the increase in voltage in the second step preferably being between 30 and 1000 V/minute.

29. A process according to any one of the claims 25-28 wherein the maximal voltage applied is about 50,000 V/m, preferably up to about 20,000 V/m, such as 10,000, 5,000 or
5 3,000 V/m.

30. A process according to any one of the claims 25-29 wherein the voltage applied is applied in at least a first and a second step, the voltage in the first step being raised continuously and the voltage in the second step being raised faster than the voltage being raised in the first step.

10 31. A process according to any one of the preceding claims wherein at least one biocomponent is isolated, said process comprising the further step of collecting the isolated biocomponent, preferably by cutting the sheet-like substrate into sections, and washing out the one or more substrate sections comprising the biocomponent to be isolated.

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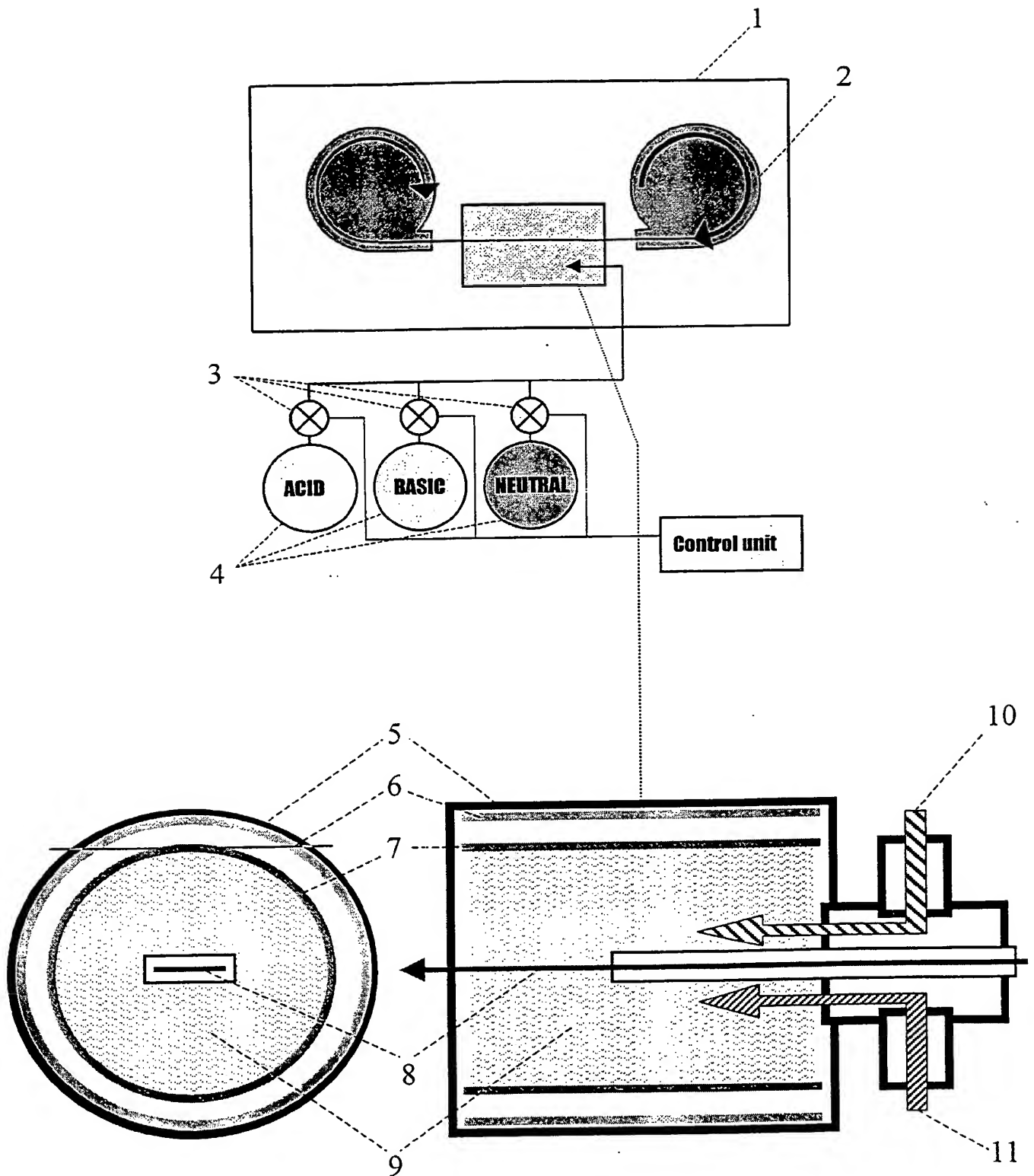


Fig 1.

Fig 2

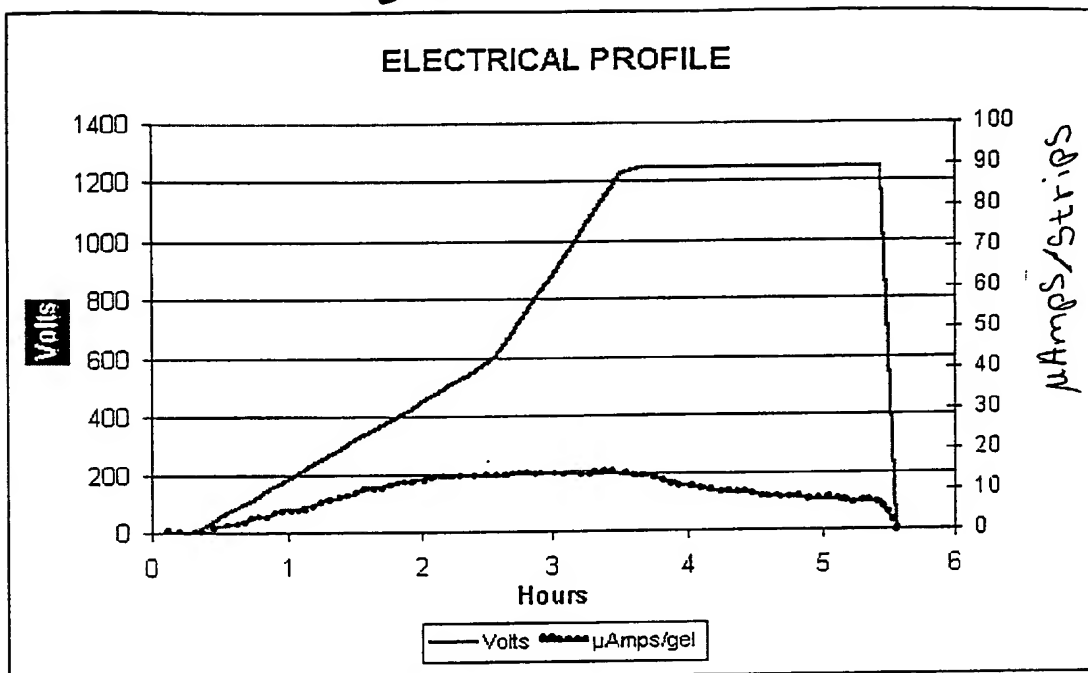
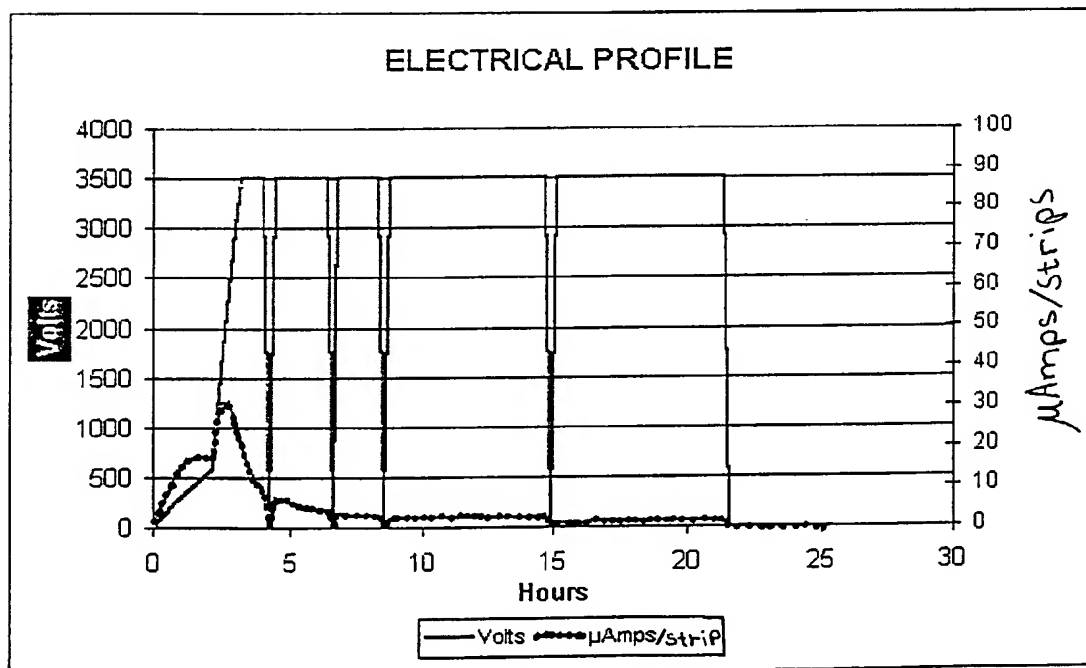


Fig 3



INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 02/00150

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N27/26 C07K1/00 G01N30/00 G01N33/00 B01J20/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C07K B01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, PAJ, CHEM ABS Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 280 567 A (FUJI PHOTO FILM CO LTD) 31 August 1988 (1988-08-31) page 5, line 29 - line 35 page 6, line 14 - line 21 ---	1-10, 13-31
X	SHYH-HORNG CHIOU ET AL : "Evaluation of commonly used electrophoretic methods for the analysis of proteins and peptides and their application to biotechnology." ANALYTICA CHIMICA ACTA, vol. 383, 1999, pages 47-60, XP002902508 the whole document ---	1-10, 13-31
E	WO 02 32591 A (PICOSEP AS) 25 April 2002 (2002-04-25) the whole document -----	1-31

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

3 June 2002

Date of mailing of the international search report

01.07.02

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INTERNATIONAL SEARCH REPORT

national application No.
PCT/DK 02/00150

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: **1-31 ,partially**
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-31 ,partially

Present claims 1-31, in rather general terms, refer to a process for separation of biocomponents, wherein a material comprising a substrate that has a surface separation layer is used. The expression "surface separation layer" is vague and unclear, and the claimed process can be performed in an extremely large number of different ways. Dependent claims 2-31 do not support any useful limitation for search as they all relate to well known features in the field of separation processes.

Furthermore, support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found for a very small proportion of the different processes claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Consequently, the search has been carried out for those parts of the claims that appear to be supported and disclosed, namely those parts related to the methods performed in example 4-5 in the description. The search has therefore been focused on a method for performing electrophoresis in order to separate proteins, using a substrate that has a plasma-polymerised layer on its surface. The plasma-polymerised layer comprises a pH gradient and can for example be built from monomers of acrylic acid and allylamine, or of acrylic acid and ethylenediamine.

The search for the method described in the specific examples 4-5 did not reveal any documents of particular relevance. Since the wording of the present claims 1-31 is extremely general, the cited documents are but examples of documents that are considered to fall within the scope of the claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK 02/00150

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0280567	A	31-08-1988	JP 1930885 C	12-05-1995
			JP 6060886 B	10-08-1994
			JP 63210653 A	01-09-1988
			JP 63210654 A	01-09-1988
			DE 3881905 D1	29-07-1993
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			US 4844786 A	04-07-1989
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WO 0232591	A	25-04-2002	WO 0232591 A2	25-04-2002
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